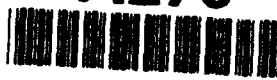


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ULTRASTRUCTURAL, CYTOCHEMICAL AND MORPHOMETRIC
ANALYSIS OF LIVER AND KIDNEY

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) The primary objective of this investigation was to determine the ultrastructural effects of diethylnitrosamine (DENA) and trichloroethylene (TCE) on hepatic and renal tissues of the Japanese medaka, <i>Oryzias latipes</i> . The study was also designed to ascertain whether DENA can function as an inducer of cellular transformation for TCE. One group of 60 medaka was exposed to DENA (10mg/L) for 48hrs, rinsed and maintained in fresh water. Another group of fish was exposed continuously to TCE (10mg/L) until sacrificed at one of the scheduled time points. A third group was exposed to DENA (10mg/L) for 48hrs, rinsed in fresh water and subsequently exposed continuously to TCE (10mg/L) until the time of sacrifice. A control group of medaka was maintained in fresh water. The sacrifices were conducted at 8, 16, 32, and 52 weeks subsequent to the initial exposure to DENA. A majority of the fish maintained for the 52 week period died prior to sacrifice. Consequently, the remaining fish were subjected only to an ultrastructural analysis. The general cellular response to DENA appeared to be consistent among exposed medaka examined at all time points. However, the - continued on next page -			
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number of cells affected by the toxin and degree of severity increased for those fish sacrificed 32 and 52 weeks after exposure to DENA. The affected parenchymal cells displayed altered mitochondria, endoplasmic reticulum, and nucleoli. Spongiosis hepatitis, multilocular cyst-like lesions, were detected in three livers of fish exposed to DENA. Focal sites of cellular aberrations in kidneys were detected at each of the time points. However, pronounced cellular degradation was evident only at the 52 week period. Relatively few cells of the hepatic tissues exposed to TCE demonstrated changes indicative of exposure to a toxic or carcinogenic agent. There was no discernible response to TCE among the cells comprising the kidney. The general pattern of response among hepatic tissues exposed to DENA/TCE was similar to that observed in parenchymal cells exposed to DENA. The general pattern of peroxidase localization was relatively consistent among cells examined at each of the time points. Peroxisomes were not quantified, however, there was an apparent increase in the number of these organelles among parenchymal cells of medaka exposed to both DENA and DENA/TCE. Peroxidase activity was also detected in bile pre-ductile cells. Definitive statements regarding acid phosphatase activity are not possible because of the inconsistency of labeling. Morphometric analysis were conducted on ratios of nuclear to cytoplasmic area and lipid to cytoplasmic area. The nuclear to cytoplasmic ratio increased for parenchymal cells exposed to either DENA or TCE, however, the values were not significant. Although not statistically significant, the lipid content of parenchymal cells exposed to DENA was less than that of control cells. The amount of lipid associated with parenchymal cells of medaka exposed to TCE was similar to that of control tissue.

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William D. Dufre 5/20/93
PI - Signature Date

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INTRODUCTION

The primary methods currently established for analyzing the carcinogenicity of chemicals are tests on mammals such as mice and rats. However, such investigations require substantial laboratory space and research funds and involve prolonged periods of time. These features, in essence, preclude expanded efforts to search for carcinogenic compounds among the various substances present in the environment. Since there appears to be a strong correlation between mutagenicity and carcinogenicity, an effective and reproducible method of testing for mutations has been developed in bacteria (Ishidate, 1980). However, there are significant differences between bacteria and mammals with regard to biochemical pathways and cellular responses to xenobiotics. Appropriately, there is a need to develop an acceptable intermediate model, representative of a position between bacteria and rodents, for the analysis of potential chemical carcinogens in man.

Investigators representing a variety of disciplines currently employ fish models as routine and reliable bioassay testing systems for the detection of toxins and/or carcinogens in the environment, and more appropriately, as rapid indicators of carcinogenicity (Masahito et al., 1988; Harada et al., 1988; Klaunig et al., 1979). Several species of fish have exhibited relative degrees of susceptibility to a variety of carcinogens. The Japanese medaka, Oryzias latipes has served as a popular model for carcinogenicity studies, many of which have included diethylnitrosamine (DENA), a site-specific carcinogen for hepatic

neoplasms and methylazoxymethanol-acetate, a compound capable of inducing neoplasms in the liver, pancreas and kidney (Hinton et al., 1988; Harada et al., 1988; Norton & Gardner, 1987; Kyono, 1978). The acquisition of basic histological and ultrastructural information has provided some knowledge into the structural changes associated with neoplasms. However, the precise mechanism of transformation and the events associated with the development of pre-neoplastic lesions, including relative metabolic processes, uptake of molecules and DNA interactions are not completely understood.

This study is designed to determine the ultrastructural and selected cytochemical effects on the liver and kidney of an initial acute exposure of medaka to DENA followed by a chronic exposure of the fish to trichloroethylene (TCE). Another objective of the investigation is to analyze the effects of DENA and TCE, independently, on hepatic and renal tissue of the medaka subsequent to acute and chronic exposures, respectively. A critical phase of the study is to determine whether DENA can function as an initiator or inducer of carcinogenesis in combination with TCE. The phenomenon of tumor promotion illustrates the synergistic interaction of cancer-inducing agents. The two stage theory of cancer formation requires the initial exposure of tissue to a carcinogen, the initiator and the subsequent presence of a promoter, a compound which is not carcinogenic when acting independently. Initiators may function as complete carcinogens and induce tumor formation when not acting in concert with another compound.

Trichloroethylene was selected as a compound for study because of its potential as an environmental contaminant. The chemical is a constituent of numerous toxic waste sites and is considered a serious risk to aquifers. Virtually no information is available on the synergism which may result from the combining of TCE and other toxic substances.

The two enzyme systems selected for examination, acid phosphatase and peroxidase are detected by means of electron--dense visual markers. Pre-neoplastic lesions and hepatocellular carcinomas which develop subsequent to the administration of DENA have demonstrated altered activity of several enzymes, including acid phosphatase (Fischer et al., 1983). Long term treatment of rodents with peroxisome proliferators such as benzafibrate has been shown to induce malignant tumors (Fahimi et al., 1982). Procedures have been developed which allow the formation of ultrastructural markers that indicate the presence of enzymes, including peroxidase. In the event such enzymes are concentrated within membrane bound bodies, a quantitative analysis of the organelle can be conducted and any subsequent changes which result from exposure to carcinogenic or cytotoxic agents can be determined.

MATERIAL AND METHODS

Medaka cultures were maintained at the U.S. Army Biomedical Research and Development Laboratory, Fort Detrick, Frederick, Maryland. Sixteen day old medaka were divided into six major groups of approximately sixty fish each. One group of fish was exposed to DENA (10 mg/L) for 48 hours in a sealed container. This concentration was selected from range finding studies and is near the solubility limit for DENA. The fish were then rinsed and held in a 10 gallon tank with a flow rate of 100 ml/min. Another group was exposed continuously to TCE (10 mg/L) for the duration of the experiment. A third group was exposed to DENA (10 mg/L) for 48 hours in a sealed container, rinsed in fresh water and exposed continually to TCE (10mg/L) until the appropriate period of sacrifice. The latter two groups of fish were maintained in 10 gallon tanks with a flow rate of 100 ml/min. Trichloroethylene was added to the tanks at a rate to ensure a constant concentration of 10 mg/L. The remaining medaka (control groups) were housed in 10 gallon tanks containing fresh water.

All fish were reared on a photoperiod regime of 16h light and 8h darkness. The feeding schedule included Tetramin twice daily, ocean plankton 4 times a week and brine shrimp 3 days a week. Nematodes were also a component of the diet with the amount given dependent upon the age of the fish.

At 8, 16, 32 and 52 weeks subsequent to the initial exposure to TCE, fifteen fish from each group were shipped to Southeastern Louisiana University and received within 24 hours. Within 48

hours of the final exposure to DENA the fish were anesthetized with tricaine methanesulfonate and the livers and kidneys excised. Ideally, ultrastructural investigations of the liver would dictate fixation by perfusion. However, the relatively small size of medaka, approximately 4 cm, has hindered the development of a reproducible and effective method of fixation by perfusion. Consequently, fixation was conducted by immersion. The tissues were processed according to the following protocols.

Transmission Electron Microscopy

Sections of tissue to be examined by conventional transmission electron microscopy were cut from each organ of interest, minced and placed in a cold solution of 0.1M sodium cacodylate (pH 7.4) buffered 4% glutaraldehyde. Entire kidneys and sections from the anterior, medial and posterior regions of the liver from each fish were processed. After 2 hrs of fixation, the tissues were rinsed several times in fresh buffer and post-fixed in cacodylate buffered 2% osmium tetroxide for 1 hr. The tissues were rinsed in buffer, dehydrated in a graded series of ethanol and embedded in POLY/BED 812. Thin-sections approximately 80 nanometers thick were cut with an ultramicrotome and stained with lead citrate and uranyl acetate. Tissues were examined and photographed with a JEOL 100S transmission electron microscope at 80kV.

Scanning Electron Microscopy

Liver samples selected for analysis by scanning electron

microscopy were fixed for 2 hrs in a 0.1M sodium cacodylate buffered (pH 7.4) solution of 4% glutaraldehyde. Subsequent to several rinses in fresh buffer, the tissues were post-fixed for 2 hrs in cacodylate buffered 2% osmium tetroxide. The tissues were rinsed and stored in buffer. Samples selected for further processing were dehydrated in a graded series of ethanol, critical-point-dried with liquid carbon dioxide and coated with a thin layer of gold by an ion sputtering unit. The samples were examined and photographed with an AMRAY 1200B scanning electron microscope at 30kV.

Cytochemistry

All methods chosen for the cytochemical analysis of tissues have been described by Lewis and Knight (1986). Tissues selected for cytochemical analysis were cut into 50 micron thick sections by means of a vibratome. Controls for all cytochemical evaluations were represented by tissues placed in an incubation medium which lacked the appropriate substrate.

Peroxidase: Portions of liver were fixed for 1 hr in a cold 0.1M phosphate buffered (pH 7.2) solution of 2% glutaraldehyde. After several washes in fresh buffer, 50 micron thick sections of tissue were cut by a vibratome and incubated for 1 hr at 37° C in a solution comprised of 0.1M phosphate buffer (pH 7.2), 0.07mM 3,3'-diaminobenzidine and 1% hydrogen peroxide. The sections were washed in buffer and post-fixed for 1 hr in phosphate buffered (pH 7.2) 2% osmium tetroxide. The specimens were rinsed in buffer, dehydrated in ethanol and embedded in POLY/BED 812. The

tissues were thin-sectioned, stained in solutions of uranyl acetate and lead citrate and examined with a transmission electron microscopy at 80kV.

Acid phosphatase: Sections of liver were fixed for 1 hr in a cold 0.06M sodium cacodylate buffered (pH 7.2) solution of 2.5% glutaraldehyde. The specimens were rinsed several times in buffer and subsequently cut into 50 micron thick sections by a vibratome. The tissues were incubated for 1 hr at 37° C in a solution comprised of 1.25% sodium glycerophosphate, 2% magnesium chloride, 0.2M Tris/maleate buffer (pH 9.0), 1% lead nitrate and distilled water. After several rinses in buffer, the tissues were post-fixed for 1 hr in a cacodylate buffered (pH 7.0) solution of 1% osmium tetroxide. The tissues were rinsed, dehydrated and embedded in POLY/BED 812. Thin-sections were cut and stained in solutions of uranyl acetate and lead citrate. The tissues were examined and photographed with a transmission electron microscope at 80kV.

Morphometry

Morphometric analysis of hepatic tissues was conducted with a Bioquant System IV program in conjunction with a compatible digitizing tablet. Five medaka from each of the experimental groups (DENA, DENA/TCE and TCE, respectively) at the 32 week period and a control group were selected for morphometric analysis. Five blocks of hepatic tissue from each fish were processed for morphometry, while five thin-sections from each block were chosen for analysis. From each section, five areas

were randomly selected and photographed at 2,000X. The electron micrographs were enlarged to 5,500X and used to determine the nuclear to cytoplasmic area of parenchymal cells and the relative area of lipid droplets per unit of parenchymal cytoplasm. The statistical program SYSAT was used for the statistical analysis of data obtained from the micrographs. A value of $p<0.05$ was accepted as statistical significance.

RESULTS

A significant number of fish from each of the tanks that contained exposed organisms died prior to the final sacrifice. In fact, only 2 fish that experienced an acute exposure to DENA survived for the 52 week period, while 3 fish in each of the TCE and DENA/TCE tanks remained alive for the duration of the study. Fish sacrificed at the 52 week period were subjected only to a basic ultrastructural evaluation because so few specimens survived to the final sacrifice. At least 9 fish in each of the control tanks survived until the designated time of sacrifice. Chemical fixation of all tissue was adequate, with little evidence of fixation related artifacts.

The primary purpose of examining hepatic tissues by scanning electron microscopy was to investigate the cystic cavities of spongiosis hepatis. Unfortunately, spongiotic lesions were not detected in the tissues prepared for scanning electron

microscopy. Consequently, descriptions of tissue samples obtained from an analysis of scanning electron micrographs will not be presented in this report. In order to obtain hepatic tissue that is adequately preserved, especially with regard to microvilli and other surface projections, the organ of interest should be chemically fixed by perfusion. However, such a method of fixation for tissues of the medaka is precluded because of the relatively small size of the fish and its arterial system. All tissues, including those selected for scanning electron microscopy, were fixed by immersion. Since ultrastructural information obtained from tissues prepared by immersion fixation and observed by scanning electron microscopy would be of limited value, this report will include only specimens examined by transmission electron microscopy.

Control Tissue - Liver

The liver of control medaka was comprised of hepatocytes aligned in a tubulosinusoidal pattern. Plates 2 cells thick extended out from a central vein. The space of Disse, attenuated endothelial cells and the sinusoidal lumen were positioned at the basal region of hepatocytes. Bile productular cells formed significant segments of intralobular biliary ductules.

Large, centric nuclei of hepatocytes contained significant quantities of euchromatin (Fig. 1), while nucleoli were distinguished by clusters of granular elements separated by anastomosing nucleolonemas. Lipid droplets, lysosomes and peroxisomes were dispersed throughout the cytoplasm. Cylindrical

mitochondria were characterized by short, narrow cristae and an electron-opaque matrix. The rough endoplasmic reticulum was arranged as flattened cisternae of variable length with no detectable pattern of organization.

Fat storing cells of Ito, also referred to as perisinusoidal cells and pericytes, were found occasionally in perisinusoidal recesses between parenchymal cells in the space of Disse. The elongated, convoluted nucleus was frequently indented by cytoplasmic lipid droplets. Heterochromatin tended to concentrate along the nuclear envelope. Rough endoplasmic reticulum and free ribosomes were scattered throughout the cytoplasm, while smooth endoplasmic reticulum was sparse. Relatively few mitochondria were detected, however, intermediate filaments were prominent. Secretory vesicles were usually present in close proximity to the plasma membrane.

Bile productule epithelial lining cells are found in the bile pre-ductules. These elongated cells possessed irregular and indented nuclei (Fig. 1). Heterochromatin was associated with the nuclear envelope. Little rough endoplasmic reticulum was evident, while large numbers of free ribosomes were dispersed throughout the cytoplasm. Relatively small, elongated mitochondria, lysosomes and secretory vesicles were randomly distributed in the cell. Microtubules and intermediate filaments were common features of most productule cells. Junctional complexes were evident between adjacent productule cells and between productule cells and parenchymal cells.

Control Tissue - Kidney

The glomerulus, a capillary network positioned within the renal capsule, consists of a tuft of convoluted loops that connect afferent and efferent arterioles. The endothelium of the capillaries is circumscribed by a basal lamina and a layer of intricate, highly branched podocytes. Small processes termed pedicles extend from the podocytes and wrap around the capillary loops by interdigitating with each other as they attach to the basal lamina.

The proximal convoluted tubule extends from the renal capsule and consists of two ultrastructurally distinct regions. Region I was characterized by the presence of prismatic cells with large, oblong nuclei. A well-developed centric nucleolus was located in a granular nucleoplasm. Pronounced secondary lysosomes and large vacuoles concentrated apically, while basal and lateral enfolds of the plasma membrane resulted in an extensive internal membrane system. Rough endoplasmic reticulum and free ribosomes were present in moderate concentrations. Micropinocytotic vesicles were typical cytoplasmic features. A prominent brush border consisting of relatively uniform microvilli extended into the lumen of the tubule. Junctional complexes were established along the apical zone.

Epithelial cells of region II contained distally positioned spherical nuclei. A distinct nucleolus was frequently associated with the nuclear envelope. Substantial numbers of small vesicles were located distally, while electron-dense granules of various diameters were concentrated proximally. There was no evidence of

crystallinity or internal structure among the observed granules. Free ribosomes were dispersed throughout the cytoplasm and cylindrical mitochondria tended to concentrate proximally. No secondary lysosomes were noted. A brush border was present, as well as junctional complexes along the cellular borders.

Tissues Exposed To DENA

The general cellular response to DENA appeared to be consistent among exposed fish examined at all time points. However, the number of cells affected by the toxin and degree of severity increased for those fish sacrificed 32 and 52 weeks after exposure to DENA. Medaka observed from the 8 and 16 week periods displayed relatively few effects of toxicity. Occasionally, focal sites of cellular alteration were detected. Many of the affected parenchymal cells exhibited a cytoplasm characterized by an increase in electron density. The cells were distinguished by prominent secondary lysosomes, autophagic vacuoles, multivesiculated bodies and lipofuscin bodies.

One of the more pronounced nuclear changes detected in parenchymal cells from fish examined after 32 and 52 weeks was a dissociation of the outer nucleolonema and dispersion of the granular elements. Several of the affected cells displayed moderate to extensive dilation of cisternae. Many of the swollen cisternae were characterized by a reduction in the number of attached ribosomes. Peroxisomes and lysosomes appeared normal in ultrastructure. Swollen mitochondria with vesiculated cristae were evident in many of the cells.

Livers of medaka analyzed at 32 and 52 weeks post-exposure to DENA frequently displayed clusters of small to medium sized cells distinguished by cytoplasms of low electron density (Fig. 2). The pleomorphic nuclei usually exhibited a high euchromatin/heterochromatin ratio (Fig. 3). The cells possessed relatively few cisternae of rough endoplasmic reticulum and substantial quantities of Golgi complexes (Fig. 4), vesicles and lysosomes. Peroxisomes, as monitored by electron-dense markers, were not a detectable cytoplasmic feature. Bile productular cells were not observed in the cellular clusters comprising these regions.

The two livers investigated from fish sacrificed at the 52 week period were characterized by a relatively large number of parenchymal cells that possessed pronounced secondary lysosomes and Mallory bodies (Fig. 5). Significant regions of the structural organization of the liver was disrupted by an influx of pleomorphic cells, the majority of which exhibited numerous, well developed Golgi complexes. The intercellular spaces contained substantial amounts of collagen (Fig. 3).

Two livers examined from the 32 week period and one liver from the 52 week period developed spongiosis hepatis, a multilocular cyst-like lesion (Fig. 6). The structural framework of the spongiotic lesions was comprised of cells characterized by elongated cytoplasmic processes and irregular nuclei. The processes of adjoining cells were maintained in position by desmosomes. Concentrated arrays of randomly positioned intermediate filaments were prominent cytoplasmic features (Fig.

7). Cells forming the lesions possessed relatively large numbers of free ribosomes, few cisternae of rough endoplasmic reticulum and a high euchromatin/heterochromatin ratio. No lipid droplets were detected.

The cells displayed numerous coated and uncoated vesicles, the majority of which were associated with the plasma membrane. There was no evidence of basement membrane-like material in the cystic zones. Numerous necrotic cells were either suspended in the cystic cavities or associated with the surrounding septa. Fibrous or flocculent material was present in many of the cavities. The concentration of flocculent material in each cavity varied. Hepatocytes located in proximity to the spongiotic lesions contained numerous autophagic vacuoles, multivesiculated bodies, Mallory bodies and amyloid deposits. Several of these features are indicative of degenerative traits. Many of the hepatocytes in this region were characterized by a loss of glycogen, fragmented endoplasmic reticulum and increases in lipid content. Pyknotic nuclei were also observed occasionally.

There was no evidence of structural aberrations in either bile productule cells or Ito cells among the livers examined at each of the time points. Cells comprising the structural framework of spongiotic lesions were similar, ultrastructurally, to Ito cells. However, there was no indication as to how the Ito cells may have arranged to form the foundation of the lesion.

Cellular responses of the kidney to DENA exposure was not as extensive or consistent as that of the liver. Focal sites of cellular aberrations were detected at each of the time points.

However, pronounced cellular degradation was evident only at the 52 week period.

There was no observable indication of neoplasia in any of the renal tissue analyzed. Indicators of cytotoxicity were especially pronounced in region II of the proximal tubule. Several of the tubules examined appeared distended as the result of the accumulation of cellular debris within the lumen. The debris included granular and flocculent material, myeloid figures, fragmented membranes, vesicles, and isolated organelles (Fig. 8).

Relatively large apical blebs protruded from cells into the tubular lumen at various sites along region II. Cytoplasm within the blebs was of low electron density with few organelles. The apical plasma membrane of several affected cells was fragmented. Cells were also observed with pyknotic nuclei, fragmented nuclear envelopes and condensed chromatin.

Cilia of cells from regions I and II exhibited several features of toxicity, including fragmentation of the plasma membrane and disorganization of microtubules. A pronounced loss of microvilli was also evident. There appeared to be a reduction in the content of electron-dense granules among the cells of region II as compared to control tissues. No structural aberrations of the granules were detected.

Tissues Exposed to DENA/TCE

The general pattern of response to the two compounds was somewhat similar to that described for hepatic tissues exposed

exclusively to DENA. The extent of cellular response increased at each of the progressive time points. However, the majority of parenchymal cells displayed no discernible effects of exposure. Isolated hepatocytes and focal zones consisting of cellular clusters were characterized by specific indicators of cytotoxicity. The most pronounced nuclear alteration concerned a dissociation of the outer nucleolonema and a concomitant dispersion of granular elements. Structural changes of rough endoplasmic reticulum included moderate to extensive dilation of cisternae. The swollen cisternae were characterized by a reduction in the number of attached ribosomes. Parenchymal cells frequently displayed cleft-like zones. Many hepatocytes in proximity to sinusoids transformed into elongated cells with swollen mitochondria (Fig. 9). Vesiculated cristae and myeloid figures also were evident in many of the affected cells.

Several of the livers displayed distinct regions that consisted of small to medium sized cells of an uncharacterized nature. The pleomorphic nuclei usually expressed a high euchromatin/heterochromatin ratio. The cells possessed relatively few cisternae of rough endoplasmic reticulum and substantial quantities of Golgi complexes, vesicles and lysosomes. Large amounts of collagen fibers were dispersed among the intercellular spaces. Peroxisomes, as expressed by cytochemical markers, were not a consistent cytoplasmic feature of the cells comprising these regions. Bile productular cells were not detected in the cellular clusters described above. However, such cells appeared to be structurally normal in areas of the liver not visually

affected by DENA.

Spongiotic lesions (Fig. 10) were detected in three livers of medaka sacrificed at the 32 week of exposure, while none were observed at the other time points. The multilocular cyst-like lesions were similar in structural framework to those examined in DENA exposed fish. The structural framework of the lesions was formed by attenuated cells with small irregular nuclei. The plasma membrane was extended to generate cytoplasmic processes. Concentrated arrays of randomly positioned intermediate filaments were prominent cytoplasmic features. The processes of adjoining cells were maintained in position by desmosomes. The cystic cavities contained fibrous or flocculent material.

There was no observable evidence of neoplasia in the kidneys of medaka exposed to DENA/TCE. Cytotoxicity was evident along the length of the proximal tubule. However, the more pronounced indicators of cellular necrosis and alteration were localized predominately in region II. The majority of proximal tubules examined appeared markedly distended as a result of the accumulation in the lumen of cellular debris, including myeloid figures, membrane fragments and isolated organelles. Large apical blebs protruded from cells into the tubular lumen at focal sites along the length of region II. Focal sites of the apical plasma membrane appeared fragmented. Many of the cells displayed pyknotic nuclei.

Tissues Exposed to TCE

Relatively few cells of the hepatic tissues exposed to TCE

demonstrated changes indicative of exposure to a toxic or carcinogenic agent. Parenchymal cells occasionally displayed evidence of cytotoxicity. The affected cells were characterized by swollen mitochondria, pronounced secondary lysosomes, and autophagic vacuoles. There were no detectable nuclear changes among the parenchymal cells observed. Bile productule cells and Ito cells appeared normal. One lesion indicative of spongiosis hepatitis was evident in the livers examined at the 52 week period of exposure. The structural features of this lesion were similar to those described for comparable aberrations detected in livers exposed to DENA and DENA/TCE.

There was no discernible response to TCE among the cells comprising the kidney. Glomeruli and renal capsules displayed no signs of cytotoxicity. The brush border of the proximal convoluted tubule was uniform in appearance, while the individual cells of the tubule displayed normal ultrastructure.

Cytochemistry - Peroxidase Localization

The general pattern of peroxidase localization was relatively consistent among all cells examined for each of the time points. Peroxisomes were identified by the presence of electron-dense material generated, indirectly, as the result of peroxidase activity. The oxidation of diaminobenzidine resulted in the formation of an intermediate compound which, subsequently, reacted with osmium tetroxide to produce a detectable precipitation. Spongiotic lesions were not detected in any of the tissue samples selected for analysis of peroxidase and acid

phosphatase activity.

Peroxisomes were not quantified because of an inconsistency among the tissue samples. Several of the specimens did not display the presence of electron-dense material which normally would be expected of peroxidase activity. The procedure selected for this investigation has proven to be very reliable for mammalian systems; however, the results indicate the peroxidase system in fish may not provide the same consistent pattern of localization demonstrated in a rodent model system. When peroxisomes were indicated by electron-dense markers, the general observations indicated an apparent increase in the number of these organelles among parenchymal cells of medaka exposed to both DENA and DENA/TCE. The localization and general appearance of detectable reaction product, indicative of peroxidase activity, was relatively consistent among the parenchymal cells examined from medaka exposed to DENA. Fish exposed solely to TCE exhibited peroxidase activity that was similar to control tissue.

Peroxidase activity in parenchymal cells, as monitored by the diaminobenzidine procedure was associated primarily with discernible peroxisomes which appeared to be randomly distributed in the cytoplasm. The internal contents of peroxisomes consisted of particulate material which was present in various concentrations (Figs. 11 & 12). Many of the peroxisomes were in contact with both smooth and rough endoplasmic reticulum, although there was no apparent continuity between the membranes forming these organelles. Peroxisomes were also associated with lipid inclusions and mitochondria.

Reaction product was also evident within the narrow channels formed between the folded membranes comprising the cristae of mitochondria. Additional reaction product was detected frequently along the surface of lipid inclusions. Reaction product was absent from the nucleus, Golgi complexes and the plasma membrane of parenchymal cells.

Peroxidase activity was detected in bile pre-ductule cells (Fig. 13). Reaction product was normally concentrated in irregularly shaped, membrane-bound vesicles. The vesicles appeared randomly distributed in the cells; however, they tended to cluster, occasionally, in regions adjacent to the nucleus. There was no evidence of peroxidase activity along cellular junctions. Peroxidase activity was detected in each pre-ductule cell examined.

Cytochemistry - Acid phosphatase Localization

The procedure used for detecting acid phosphatase activity was significantly inferior to that employed for the localization of peroxidase reaction product. In many instances lead precipitate, the visual marker employed in the protocol selected for this investigation, was detected in regions of the cell known not to possess acid phosphatase activity. Although the distribution of label was not consistent among the parenchymal cells analyzed, discernible reaction product was frequently associated with structures whose ultrastructural features were characteristic of lysosomes (Fig. 14). Because of the inconsistency of labeling, definitive statements concerning the

distribution and quantification of acid phosphatase containing sites in parenchymal cells would be misleading and erroneous. There was no discernible acid phosphatase activity detected among bile pre-ductule cells or along the surface of microvilli which formed the bile pre-ductules.

Morphometry

Morphometric analysis were conducted on parenchymal cells of hepatic tissues excised from medaka exposed to either TCE or DENA and sacrificed at the 32 week period (Tables 1 & 2). The protocol originally was designed for a morphometric analysis of tissues obtained at 52 weeks post exposure to DENA. However, as a result of extensive mortality among the fish by 52 weeks, sufficient numbers of medaka were not available for examination. Consequently, morphometric data will be presented only for the 32 week period.

The measurements are calculations of ratios of nuclear to cytoplasmic area (μm^2) and lipid to cytoplasmic area (μm^2) of the parenchymal cells. Nuclear to cytoplasmic ratios increased for parenchymal cells exposed to either DENA or TCE; however, the values were not significant because of the large variation among fish of the control group. Medaka exposed to DENA experienced a greater increase in nuclear to cytoplasmic ratio than the fish exposed to TCE. Although not statistically significant, the lipid content of parenchymal cells exposed to DENA was less than that of control cells. The amount of lipid present in parenchymal cells of medaka exposed to TCE was similar to that of control

tissue.

CONCLUSIONS

The results of this investigation suggest TCE, at a chronic exposure level of 10mg/L does not consistently induce detectable ultrastructural changes in parenchymal cells until after 32 weeks of exposure. The morphometric data obtained from tissues examined from the 32 week period indicates a very limited response of parenchymal cells to TCE. Nuclear to cytoplasmic ratios and the lipid content of parenchymal cells were similar to values obtained from control tissue. The cellular aberrations which were examined in tissues processed at the S2 week period tended to be focal in distribution and cytotoxic in nature.

Spongiosis hepatis was detected to a limited degree in hepatic tissues from each of the three exposed groups of medaka. Spongiosis hepatis, a hepatic lesion characterized by multiple cyst-like cavities was described initially in rats by Bannasch et al. (1981). The condition rarely occurs spontaneously, but rather develops primarily in response to hepatocarcinogens. The structural foundation of spongiotic lesions consists of cells that are comparable, ultrastructurally, to perisinusoidal cells of the liver (Hinton et al., 1988; Couch and Courtney, 1987; Bannasch et al., 1981).

Investigators have reported that rats exposed to the carcinogen N-nitrosomorpholine possess copious amounts of

basement membrane-like material and bundles of collagen fibers in cells which form the walls of spongiotic lesions (Bannasch et al., 1981). Hinton et al. (1984) observed the presence of basement-like material in spongiotic livers of medaka exposed to methylazoxymethanol acetate. There was no indication of collagen or basement membrane-like material in the cystic cavities of spongiotic lesions examined during this investigation. Variations in the presence or absence of collagen and basement membrane-like material may reflect differential binding of the carcinogens to DNA and/or critical regulatory proteins.

There have been relatively few descriptions of definitive ultrastructural changes that occur in perisinusoidal cells of fish as a result of exposure to hepatocarcinogens, although investigators have implied the perisinusoidal cell as the cell type which forms the structural framework of spongiosis hepatis. The ultrastructural information obtained from this investigation indicates DENA and/or its metabolites induce chronic changes in perisinusoidal cells with regard to protein synthesis and gene activity as manifested by increases in ribosomes and euchromatic/heterochromatin ratios. The cells also demonstrate stimulated micropinocytotic activity. Whereas micropinocytotic vesicles are uncommon in perisinusoidal cells of control liver, comparable cells associated with spongiotic lesions display an abundance of the structures.

This final report is based, solely, on observations made from tissues processed for electron microscopy. Several fish from each of the experimental and control groups were selected

for histological analysis. These fish were placed in Bouin's fixative and shipped to the U.S. Army Biomedical Research and Development Laboratory for processing. Since the Principal Investigator had not yet received the histopathology report at the time this final report was written, all results and conclusions were based on information obtained from relatively small numbers of animals and random samples from each organ.

A critical objective of this investigation was to determine whether DENA can function as an initiator or inducer of carcinogenesis in combination with TCE. The two stage theory of carcinogenesis requires an initial exposure of tissue to an initiator, a carcinogen, and subsequent contact with a promoter, a compound which is not carcinogenic when acting independently. Although pronounced cellular changes were noted during the ultrastructural observation of exposed tissues, neither malignant nor benign tumors were detected. The results of this study indicate TCE, independently or after an initial exposure to DENA, consequently, it appears the hepatocarcinogen DENA, at a concentration of 10 mg/L does not function as an initiator or inducer of carcinogenesis in combination with TCE. This deduction is based solely on a limited number of ultrastructural observations. Fish processed for histological analysis were not available to the Principal Investigator prior to the preparation of this report. The factor of concentration should be considered during this discussion. Although discernible systemic lesions were not generated in hepatic tissues exposed to TCE exclusively or neoplasms were not produced in livers treated with both DENA

and TCE, the reason could relate to an insufficient initiating concentration of DENA and/or an inadequate concentration of TCE to elicit the desired effects. Additional studies should be conducted to determine the ultrastructural effects of various concentrations of TCE, both independently and in combination with DENA on hepatic tissue of the medaka.

The increase in nuclear-to-cytoplasmic ratio and reduction in lipid content of many of the hepatocytes exposed to DENA, although not statistically significant, does reflect detectable responses to DENA. These results are similar to those of a study designed to determine the effect of exposure of medaka liver to methylazoxymethanol acetate (Hinton et al., 1984). The study revealed a significant reduction in the lipid content of exposed hepatocytes and an increase in nuclear-to-cytoplasmic ratio. The investigators were not able to determine whether the reported alterations are hyperplastic and appear in response to repair to the toxic effect or whether they are truly neoplastic.

One objective of this investigation was to determine the capacity of either DENA or TCE to induce the proliferation of peroxisomes among hepatocytes. A cytochemical procedure which has proven to be very effective in the localization of peroxidase activity in hepatocytes of rodents was selected (Meijer and Afzelius, 1989). The ability to identify the presence of peroxidase activity with an electron-dense visual marker is important since peroxisomes are frequently mistaken for lysosomes in the absence of a definable cytochemical marker. Consequently, any numerical analysis of either organelle constructed strictly

on ultrastructural observations may be misleading and erroneous.

Several drugs with hypolipidemic effects have been demonstrated to cause an extensive proliferation of peroxisomes in the livers of rats after a few days of treatment (Hess et al., 1965). Long-term treatment of rodents with peroxisome proliferators has been shown to cause liver cancer (Reddy et al., 1982). This transformation has been attributed to the indigenous production of hydrogen peroxide in peroxisomes being increased after proliferation of peroxisomes, which results in an oxidative stress on hepatocytes (Reddy et al., 1982). Although technical problems precluded a morphometric analysis of peroxisome distribution, the results of this study appear to indicate neither DENA nor TCE are capable of inducing a significant proliferation of peroxisomes among exposed hepatocytes. The number of peroxisomes appeared slightly elevated in hepatocytes exposed to DENA, however, the variation among individual cells was substantial. The number of peroxisomes among cells exposed to TCE was similar to that of control cells.

One valuable aspect of the cytochemical procedure designed to localize peroxidase activity is its ability to identify the presence of the enzyme in structures which do not display the ultrastructural features of peroxisomes. Electron-dense reaction product denoting the activity of peroxidase was identified in irregularly shaped, membrane-bound bodies of bile productule cells. This document represents the first report of demonstrative peroxidase activity in bile productule cells. Although a morphometric analysis was not conducted on the electron-dense

bodies depicting the presence of peroxidase, bile productule cells of both control and exposed hepatic tissues appeared to possess comparable amounts of the electron-dense material. The metabolic functions of bile productule cells have not yet been investigated, consequently, the significance of this finding is presently difficult to assess.

The Japanese medaka has served as a popular fish model for carcinogenicity studies, many of which have involved DENA, a site-specific carcinogen for hepatic neoplasms (Norton and Gardner, 1987; Hawkins et al., 1986; Kyono, 1978). Very little data is available on the ultrastructural effects of DENA exposure to renal tissue of the medaka, however, renal tumors have been induced chemically in the rainbow trout by several structurally unrelated carcinogens, including dimethylnitrosamine (Grieco et al., 1978), and N-methyl-N-nitro-N-nitrosoguanidine (Hendricks et al., 1980). The relative paucity of chemically induced renal tumors in fishes may reflect an efficacious DNA repair system, which, according to Ishikawa et al., (1982) depends on both cell type and stage of development.

Dimethylnitrosamine (DMNA) is a potent renal carcinogen for the rat. Several cytotoxic changes involving the proliferation of smooth endoplasmic reticulum, increased content of lipid vacuoles and the formation of nuclear bodies were induced over a 6 week period in the epithelium of proximal convoluted tubules of rats exposed to a single dose of DMNA (Hard et al., 1984). Comparable effects were not detected in this study. However, certain degenerative features such as the presence of autophagic

vacuoles, swollen mitochondria and pyknotic nuclei were evident focally in cells exposed to DENA solely and in the cells of kidneys exposed to both DENA and TCE. Such cellular alterations appear to be consistent manifestations of exposure to both forms of the nitrosamine. Discernible evidence of cytotoxicity was not evident in the cells of kidneys exposed only to TCE.

The majority of aberrations observed in this study are characteristic of toxic insult to cells. Changes in the dimensions of microvilli associated with the epithelium of proximal tubules have also resulted from a variety of agents and/or conditions, including exposure to aromatic hydrocarbons (Norton and Mattie, 1987). The presence of autophagic vacuoles and swollen mitochondria with a clear matrix represent toxic features that are evident in the typical progression leading to cellular necrosis. The degree of degradation is extensive along region II of the proximal tubule, however, cells displaying distinct features of regeneration are evident in the compromised zones.

Results of this investigation indicate DENA or its metabolites are capable of inducing changes which result in pronounced aberrations in the epithelium of proximal tubules of medaka subsequent to an acute exposure during embryonic development. Chronic exposure to TCE, at least at the concentration chosen for this study does not result in comparable cytotoxic effects. Despite the magnitude of cellular damage evident in renal tissue exposed to DENA, the organ does appear capable of recovering from toxic insult as indicated by the

presence of regenerating cells.

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LIST OF FIGURES

Figure 1. Hepatocytes are characterized by large clusters of glycogen (GY), a spherical nucleus (NU) with pronounced euchromatin and scattered cisternae of rough endoplasmic reticulum (RER). A bile productule cell (BP) contains an indented nucleus and is in contact with a bile canalculus (BC). Control tissue. Magnification: X1-,300.

Figure 2. Pleomorphic cells with irregular nuclei and electron-lucent cytoplasm tend to concentrate along the border of spongiotic lesions. Tissue exposed to DENA for 32 weeks. Magnification: X5,300.

Figure 3. Dissociated collagen fibers (CO) are positioned along a cell with an indented nucleus (NU) and electron-lucent cytoplasm. Tissue exposed to DENA for 32 weeks. Magnification: X9,000.

Figure 4. An extensive network of Golgi complexes (GC) and vesicles (arrows) characterize the electron-lucent cytoplasm of a cell adjacent to a spongiotic lesion. Tissue exposed to DENA for 32 weeks. Magnification: X30,000.

Figure 5. A large Mallory body (MB) consists of electron-dense fibrous material and what appears to be lipid droplets (LD). Tissue exposed to DENA for 52 weeks. Magnification: X22,800.

Figure 6. Spongiosis hepatis is distinguished by pronounced multiocular cyst-like cavities (CA) which are bordered by attenuated cells (arrow). Note the presence of cellular debris and uncharacterized cells within the cavities (arrowheads). Tissue exposed to DENA for 32 weeks. Magnification: X5,200.

Figure 7. The structural foundation of spongiosis hepatis is comprised of perisinusoidal cells that possess an irregularly shaped nucleus (NU), elongated cytoplasmic extensions (arrowhead), and arrays of intermediate filaments (arrows) which extend in several directions. Tissue exposed to DENA for 32 weeks. Magnification: X30,600.

Figure 8. Cellular debris, located in the lumen of the proximal convoluted tubule, consists of flocculent material (FL) and fragments of membranes (arrows). Tissue exposed to DENA for 32 weeks. Magnification: X22,500.

Figure 9. The spatial relationship of hepatocytes has been disrupted by an influx of electron-lucent cells (LU). Elongated hepatocytes (HE) are in close proximity to a disrupted sinusoid and are characterized by swollen mitochondria (arrows). Tissue exposed to DENA/TCE for 52 weeks. Magnification: X4,300.

Figure 10. Adjacent cells that form the structural framework of spongiotic lesions are maintained in position by desmosomes (arrows). The cavities (CA) are devoid of any

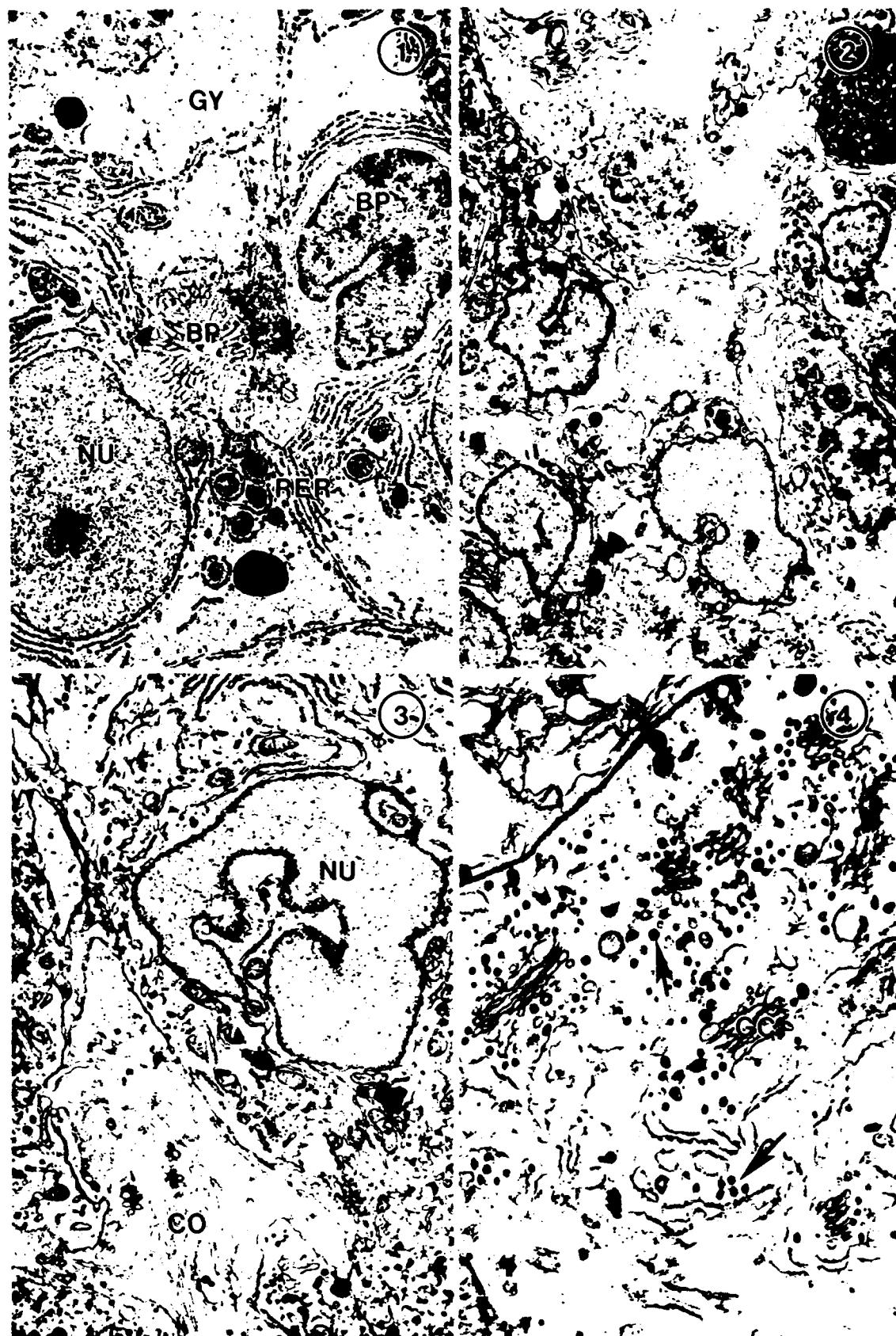
appreciable amount of material. Unidentified cell types (CE) are located in the lesion. Tissue exposed to DENA/TCE for 32 weeks. Magnification: X9,800.

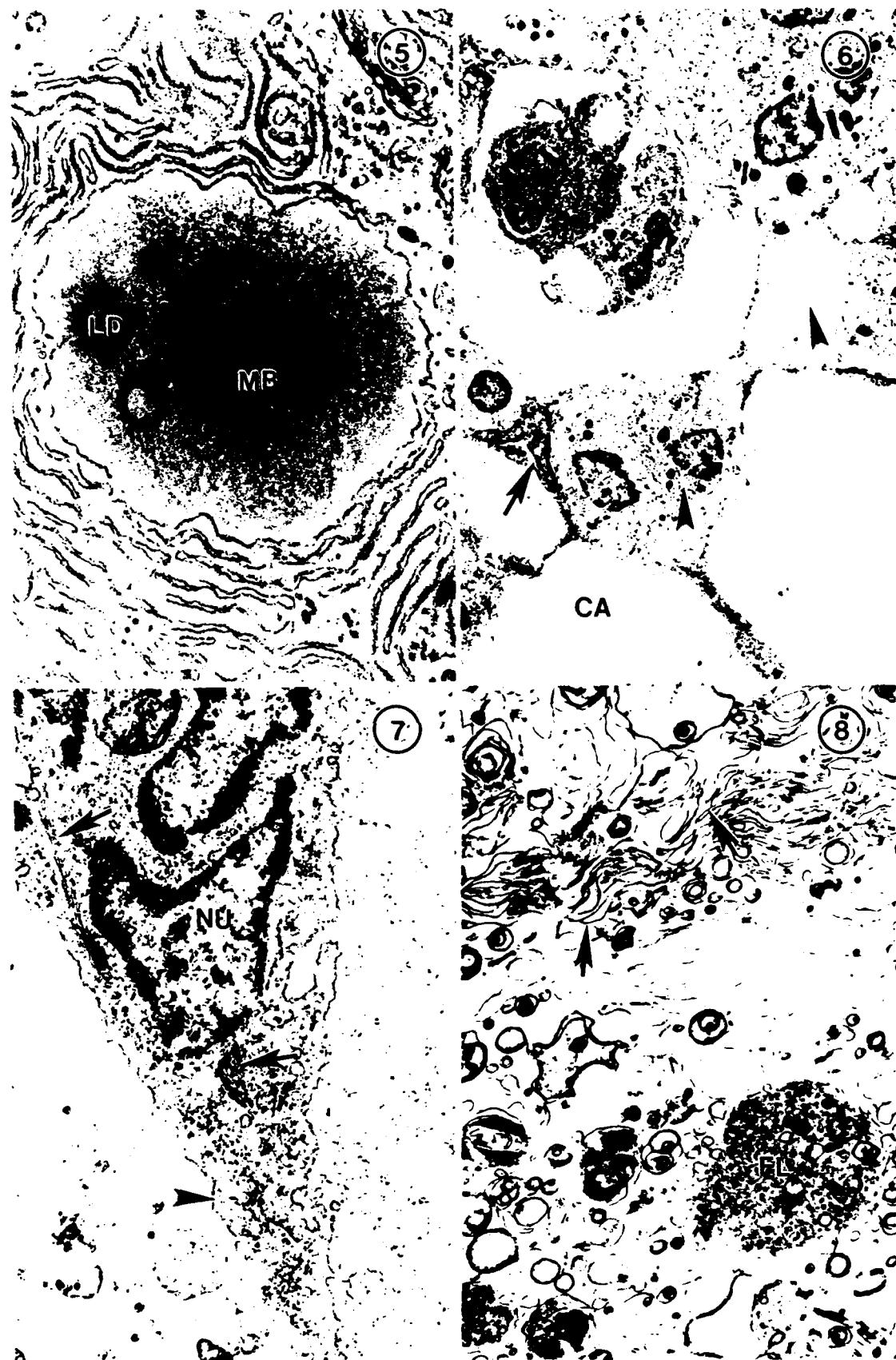
Figure 11. Peroxisomes (PE), as indicated by electron-dense material generated by peroxidase activity, are spherical membrane-bound bodies with electron-dense particulate matter. Tissue exposed to DENA for 32 weeks. Magnification: X14,000.

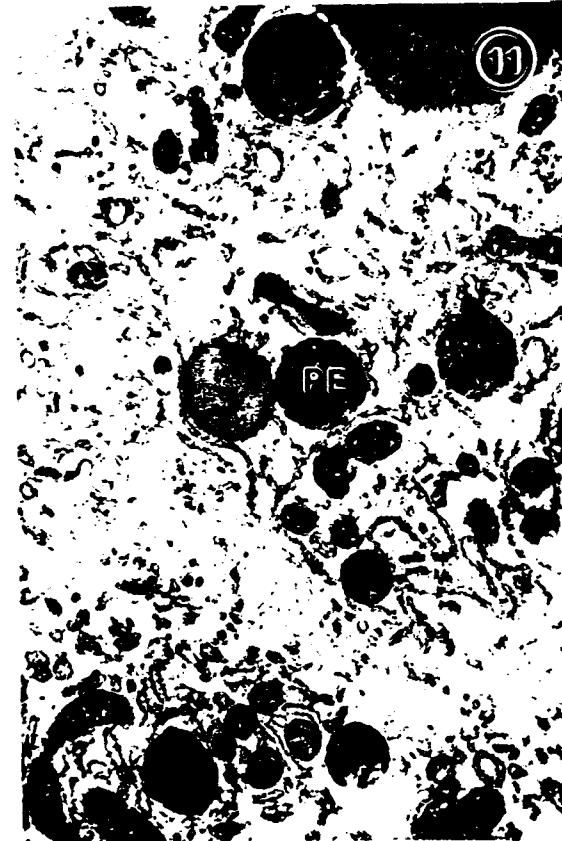
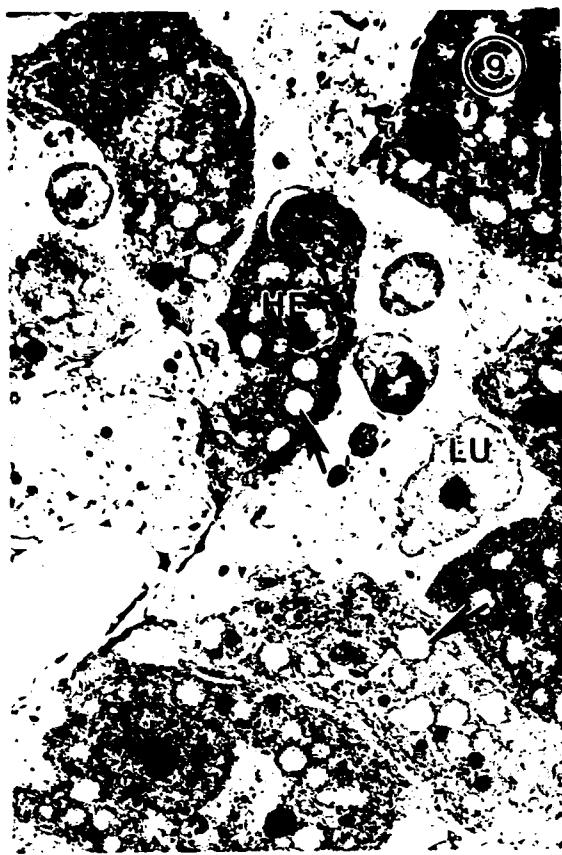
Figure 12. Several peroxisomes (PE) are located near a lipid droplet (LI) enveloped by a mitochondrion (MI). Tissue exposed to DENA for 32 weeks. Magnification X18,200.

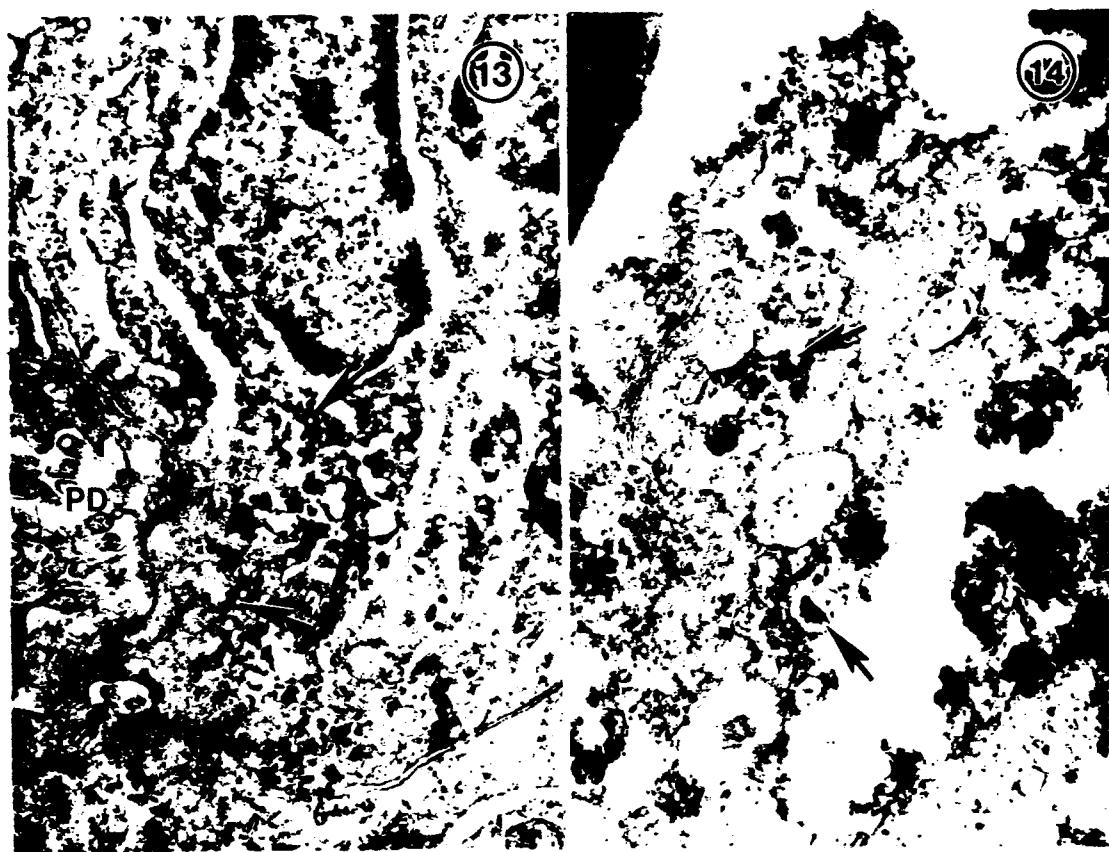
Figure 13. Electron-dense material (arrows), indicative of peroxidase activity, is present in perinuclear vesicles of a bile productule cell which lines a productule (PD). Tissue exposed to DENA for 32 weeks. Magnification: X23,500.

Figure 14. Acid phosphatase activity is indicated by electron-dense material which is concentrated, primarily, in membrane bound vesicles (arrows). Tissue exposed to DENA for 32 weeks. Magnification: X24,000.









APPENDIX

TABLE 1

Ratios of nuclei and lipid areas to cytoplasmic area, respectively, for parenchymal cells (liver) of medaka exposed to either diethylnitrosamine (DENA) or trichloroethylene (TCE) and examined at the 32 week period. Values for ratios are represented in μm^2 . Values were analyzed for significant differences at the 0.05 level of confidence (Student's t-test).

	Control	DENA	TCE
Nucleus/Cytoplasm	7.7+4.5*	11.1+3.4*	8.2+4.4*
Lipid/Cytoplasm	12.6+6.1*	7.1+3.4*	11.7+4.2*

*mean and standard deviation

TABLE 2

Ratios of nuclei and lipid areas to cytoplasmic area, respectively, for parenchymal cells (liver) of individual fish exposed to either diethylnitrosamine (DENA) or trichloroethylene (TCE). Data are also included for control fish (C). Values for ratios are represented in m^2 . Values were analyzed for significant differences at the 0.05 level of confidence (Student's t-test).

Individual Fish	Nucleus/Cytoplasm	Lipid/Cytoplasm
C-1	6.3+4.1*	11.5+4.5
C-2	7.4+5.1	12.2+7.3
C-3	6.8+4.4	18.7+8.9
C-4	9.7+3.9	13.4+3.4
C-5	8.1+4.9	17.3+6.5
DENA-1	11.4+3.8	6.1+2.9
DENA-2	10.7+3.0	8.2+3.5
DENA-3	9.9+2.5	4.2+2.8
DENA-4	12.1+3.3	7.6+3.5
DENA-5	11.5+2.9	9.5+4.1
TCE-1	7.3+4.1	10.7+3.9
TCE-2	8.9+5.1	9.8+3.3
TCE-3	7.2+4.5	11.7+4.5
TCE-4	8.6+3.5	12.9+5.1
TCE-5	9.3+4.9	13.5+4.4

*mean and standard deviation